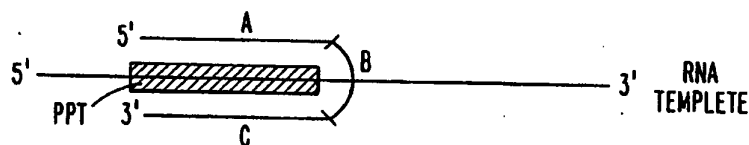


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<b>(21) International Application Number:</b> PCT/US93/09300 <b>(22) International Filing Date:</b> 29 September 1993 (29.09.93) <b>(30) Priority data:</b> 954,184 29 September 1992 (29.09.92) US <b>(60) Parent Application or Grant</b> (63) Related by Continuation US 954,184 (CIP) Filed on 29 September 1992 (29.09.92) <b>(71) Applicants (for all designated States except US):</b> APOLLON, INC. [US/US]; One Great Valley Parkway, Suite 30, Malvern, PA 19355 (US). MAX-PLANCK-GESSELLSCHAFT ZUR FORDERUNG DER WISSENSCHAFTEN E.V. [DE/DE]; Busenstrasse 10, D-3400 Gottingen (DE).		<b>(72) Inventor; and</b> <b>(75) Inventor/Applicant (for US only) :</b> MOELLING, Karin [DE/DE]; Inhestrasse 43, D-1000 Berlin 33 (DE). <b>(74) Agents:</b> ELDERKIN, Dianne, B. et al.; Woodcock Washburn Kurtz Mackiewicz & Norris, One Liberty Place - 46th Floor, Philadelphia, PA 19103-7301 (US). <b>(81) Designated States:</b> CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). <b>Published</b> With international search report.	

**(54) Title:** ANTI-VIRAL OLIGOMERS THAT BIND POLYPURINE TRACTS OF SINGLE-STRANDED RNA OR RNA-DNA HYBRIDS**(57) Abstract**

The present invention provides methods of inhibiting a virus with which a vertebrate is infected and which replicates via an RNA template comprising the administration of an antisense or triplex-forming oligonucleotide or a derivative thereof capable of binding to a polypurine-rich tract in a region of single-stranded RNA or RNA-DNA-hybrid, respectively. Chimeric oligonucleotides comprising binding domains (A and C) connected by a linker (B), and which are capable of forming triplex structures with single-stranded nucleic acids, as shown in the figure, are also disclosed.

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## ANTI-VIRAL OLIGOMERS THAT BIND POLYPURINE TRACTS OF SINGLE-STRANDED RNA OR RNA-DNA HYBRIDS

### Background of the Invention

5           This invention provides methods for inhibiting viral replication through the use of antisense and triplex-forming oligonucleotides. Antisense oligonucleotides are capable of specific hybridization with a nucleotide sequence of single-stranded RNA while triplex-forming oligonucleotides are known  
10 to be capable of specific hybridization with double-stranded DNA, double-stranded RNA, and DNA-RNA hybrids.

          Triplex helix formation with double-stranded DNA is an approach known in the art for potential sequence-specific DNA recognition. An oligonucleotide is targeted against a  
15 specific region of double-stranded DNA, resulting in the formation of a triplex. Where the triplex forming oligonucleotide (TFO) has a sufficient length, such as 8-26 nucleotides, it is expected to bind to unique sites. It has been reported that sequence specific recognition of the major groove of DNA  
20 at homopurine.homopyrimidine sequences is achieved by homopyrimidine oligonucleotides. Sun et al., *Proc. Natl. Acad. Sci. USA*, July, 1991, 88, 6023-6027. The TFO may be either parallel or anti-parallel to the target DNA strand. Experimental methods have been devised for detection of triple helix  
25 formation. Maher et al., *Inhibition of DNA/Protein Interactions by Oligonucleotide-Directed DNA Triple Helix Formation: Progress and Prospects in Prospects for Antisense Nucleic Therapy of Cancer and AIDS*, 227-242 (1991).

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All retroviruses and hepadna viruses, such as HIV and hepatitis, replicate by the conversion of an RNA template into DNA through the action of the enzyme, reverse transcriptase (RT). This enzyme copies the RNA template into a complementary DNA strand, giving rise to an RNA-DNA hybrid. The enzyme then degrades the RNA strand through its RNase H function and the DNA strand is copied into double-stranded DNA. Two primers are involved in the reverse transcription of viral RNA into double-stranded DNA. One of these primers is a polypurine tract (PPT) which allows initiation of the plus-strand DNA. K. Moelling, *Minireview: The RNase H of HIV-1, Med. Microbiol. Lett.* 1992, 1, 71-77. Apparently all known retroviruses such as Rous Sarcoma Virus, use polypurine tracts as primers.

Two PPT's are present in HIV; one is located within the coding region of the integrase gene and the other resides near the 3' long terminal repeat (LTR). The PPT is one of the most highly conserved regions among the known HIV-1 retroviral isolates, as illustrated in Tables 1A and 1B below. The nucleotides in the consensus sequences at which variations occur are indicated in lower case.

TABLE 1A

## 3' LTR PPT

	CONSENSUS	aAAagAAaAGGGGGGACTGGAaGGGc
	HIVLAI	A--AG--A-----A---C
25	HIVNL43	A--AG--A-----A---C
	HIVBRVA	A--AG--A-----A---C
	HIVSC	A--AG--C-----A---C
	HIVBAL1	T--GA--A-----A---C
	HIVJRCSF	A--AG--A-----A---C
30	HIVOYI	A--AG--A-----A---C
	HIVSF2	A--AG--A-----A---C
	HIVSF162	A--AG--A-----A---T
	HIVSF33	A--AG--A-----A---C
	HIVHAN	A--AG--A-----A---T
35	HIVRF	A--AG--A-----T---C

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TABLE 1B

## Integrase PPT

	CONSENSUS	<b>AAAAGAAAAGGGGGG</b> ATTGGGGGgTA
	HIVLAI	-----G--
5	HIVHXB2R	-----G--
	HIVMN	-----G--
	HIVJRCSF	-----G--
	HIVYOI	-----G--
	HIVSF2	-----A--
10	HIVNYSCG	-----G--
	HIVNL43	-----G--
	HIVHAN	-----G--
	HIVRF	-----G--

One targeted PPT consists of a sequence of 16 contiguous purines common to both the 3' LTR and the integrase gene: AAAAGAAAAGGGGGGA (SEQ ID NO: 1). The 16 contiguous purines are indicated in bold in the "Consensus" sequences above. The targeted PPT can also consist of a sequence of 26 nucleotides with all purines except for 2 pyrimidines present near the 3' end, i.e., AAAAGAAAAGGGGGGACTGGAAGGGC (SEQ ID NO: 2). The two pyrimidines are underlined in the "Consensus" 3' LTR sequence above. The PPT region near the 3' LTR is essential for HIV-1 replication, as demonstrated by a non-infectious RT/RNase H mutant which cannot initiate plus-strand DNA synthesis at the PPT *in vitro*. B. Wohrl et al., *Mutations of a Conserved Residue within HIV-1 Ribonuclease H Affect Its Exo- and Endonuclease Activities*, *J. Mol. Biol.* 1991, 220, 801-818. It has also been demonstrated that RT/RNase H specifically generates a PPT RNA primer for plus-strand DNA synthesis in HIV-1. B. Wohrl & K. Moelling, *Interaction of HIV-1 Ribonuclease H with Polypurine Tract Containing RNA-DNA Hybrids*, *Biochemistry* 1990, 29, 10141-10147.

Antisense oligonucleotides have been targeted against HIV-1 viral RNA in a site adjacent to the primer binding site which is folded into a loop. Buck et al., *Phosphate-Methylated DNA Aimed at HIV-1 RNA Loops and Integrated DNA Inhibits Viral*

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*Infectivity, Science 1990, 248, 208-212. This work did not involve the PPT, however.*

The PPT region of HIV-1 has been targeted for the formation of a triple helix. Sun et al., *Triple-Helix Formation by  $\alpha$  Oligodeoxynucleotides and  $\alpha$  Oligodeoxynucleotide-Intercalator Conjugates, Proc. Natl. Acad. Sci. 1991, 88, 6023-6027.* Sun et al. synthesized a series of oligonucleotides having a single 16-nucleotide long sequence, TTTTCTTTTCCCCCT (SEQ ID NO: 5), which were capable of binding the double-stranded DNA HIV PPT region *in vivo*. Thus, the prior art does not provide for the formation of a duplex or a triplex involving the PPT region at a stage of viral replication other than that of double-stranded DNA. Triplex formation with double-stranded DNA occurs through binding with a DNA homopurine strand in the major groove of the DNA duplex. Sun et al. Targeting double-stranded DNA presents a major limitation for designing viral drug therapies. Double-stranded viral DNA is present mainly in the nucleus of the infected cell and it is more difficult to deliver drug therapies into the nucleus. Contrastingly, single-stranded viral RNA and RNA-DNA hybrids are predominantly found in the cytoplasm. Therapeutics targeting single-stranded RNA, or RNA-DNA hybrids, provide a significant advantage over those targeting double-stranded DNA. Furthermore, unlike double-stranded viral DNA, single-stranded RNA and RNA-DNA hybrids are present prior to provirus formation and integration. Thus, therapeutics targeting single-stranded RNA and RNA-DNA hybrids could also provide greater protection against *de novo* infection.

#### Summary of the Invention

The present invention provides methods of inhibiting RNA virus replication by the administration of an antisense oligonucleotide or a triplex forming oligonucleotide, or derivatives thereof, to a vertebrate infected with said virus wherein the oligonucleotide is capable of binding to single-stranded RNA or RNA-DNA hybrids containing a polypurine-rich tract. The present invention thus targets the earlier stages

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of viral replication before double-stranded DNA is formed. The antisense and triplex-forming oligonucleotides generally range in size from about 8 to about 54 nucleotides long, or longer, and may be modified according to methods known in the art. The oligonucleotides are capable of binding with at least a portion of the PPT region, however, the oligonucleotides may extend beyond the PPT. The nucleotides may match the PPT region exactly or may contain several mismatches.

A preferred embodiment of the instant invention provides methods for treating HIV infection whereby an effective amount of oligonucleotide, or derivative thereof, is administered to an infected vertebrate, preferably human.

In a further preferred embodiment, the present invention provides chimeric oligonucleotides capable of binding to a polypurine-rich tract of single-stranded viral RNA and forming a triplex, thereby preventing its reverse transcription into viral DNA. The chimeric oligonucleotides are of the general formula 5'-A-B-C-3' wherein segment A comprises a sequence complementary to PPT in parallel orientation, segment B comprises a linker, and segment C comprises a sequence complementary to PPT in antiparallel orientation.

#### **Brief Description of the Drawings**

Figure 1 is a schematic diagram of the constructs used in the Examples.

Figures 2a and 2b schematically depict the oligonucleotides of the present invention and how they form triplexes with single-stranded RNA.

Figures 3a and 3b depict the TFO A sequence and structure, respectively.

Figure 4 is a plot of data quantified from a phosphorimager depicting cleavage inhibition by oligonucleotides GT1, GT2, and GU1.

#### **Detailed Description of the Invention**

The present invention provides methods of inhibiting RNA virus replication by the administration of an antisense or

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triplex-forming oligonucleotide, or derivative thereof, to a vertebrate wherein the oligonucleotide is capable of binding to single-stranded RNA or RNA-DNA hybrids containing a polypurine-rich tract (PPT). The PPT serves as a primer for reverse transcription of viral RNA into double-stranded DNA and is one of the most highly conserved regions among known HIV-1 viral isolates. The PPT region is necessary for HIV-1 replication. Single-stranded RNA and RNA-DNA hybrids are structures which occur at various points in viral replication. For example, retroviruses begin with a single-stranded RNA, which is reverse transcribed into DNA, thus forming an RNA-DNA hybrid. The RNA strand is then degraded by the RNase H function of reverse transcriptase and the remaining DNA strand is duplicated to form double-stranded DNA.

Several retroviruses and viruses which replicate via an RNA template which are capable of infecting vertebrates are known, including hepatitis and HIV. The RNA viruses which are the subject of the present invention have one or more polypurine tracts (PPTs), highly conserved regions consisting essentially of purines.

One embodiment of the present invention provides an antisense oligonucleotide directed against a PPT on single-stranded viral RNA. The single strand of RNA is used by the virus as a template for reverse transcription, and one object of the invention is to interfere with viral replication at this stage through the formation of a duplex at the polypurine-rich tract. The invention thus provides methods for inhibiting pathogenesis by administering an antisense oligomer capable of forming a duplex with the PPT of the RNA strand whereby the duplex prevents elongation of cDNA synthesis. The duplex can be detected, for example, by treatment with RNase H which specifically cleaves the RNA-DNA hybrid region where the antisense oligomer is bound.

A further aspect of the present invention targets the formation of RNA-DNA hybrids during viral replication. These naturally occurring hybrids form upon reverse transcription of the RNA template into a DNA strand and these hybrids are thus



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essential for replication in RNA viruses. One purpose of this invention is to interfere with viral replication at this stage through the formation of a triplex with the RNA-DNA hybrid. The invention provides methods for inhibiting pathogenesis by  
5 administering an oligomer capable of forming a triplex with the hybrid at the PPT and thus protecting the PPT from RNase H digestion.

In yet a further aspect of the present invention, oligonucleotides directed against PPT on single-stranded RNA  
10 are provided which are capable of forming triplexes with the single-stranded RNA in the following general orientations: R-R-D or D-R-D. Oligonucleotides capable of forming D-R-R triplexes are also contemplated. The middle "R" represents the viral RNA template. The remaining "R's" and "D's" represent  
15 the "A" and "C" segments of the oligonucleotides of the invention in the general binding orientation "A-R-C" with the RNA template. See Figure 2b.

The oligonucleotides of the present invention are capable of protecting the PPT region, whether in the form of  
20 single-stranded RNA or RNA-DNA hybrid. The binding of the triplex-forming oligonucleotide to the PPT is measured by protection from RNase H digestion, since RNase H degrades RNA only when in RNA-DNA hybrids. TFO binding to a double-stranded DNA, in contrast, affords protection from DNase I digestion.  
25 Increased premature termination of cDNA synthesis at the PPT indicates antisense binding to single-stranded RNA.

Antisense or TFO binding in all of the above situations may also be measured in several other ways. For example, altered migration properties as measured by gel shift, physical  
30 chemical parameters such as melting temperatures, and by footprinting. Since the PPT region is highly conserved across known HIV-1 viral isolates, the same oligonucleotide may be administered to combat suspected infections of all of the known isolates.

35 It will be understood by those skilled in the art that PPT regions in viruses other than those specifically disclosed are contemplated. In choosing oligonucleotides which

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target PPT regions, a PPT region conserved among viral isolates can be identified by a computer-aided comparison of known nucleotide sequences among known isolates of a virus. The sequence to be identified is a polypurine sequence of at least  
5 about 8 nucleotides long, and preferably at least about 26 nucleotides long, wherein at least about 50%, and preferably about 80%, of the nucleotides are identical across the viral isolates and wherein at least about 70%, and preferably at least about 80%, and more preferably at least about 90%, of the  
10 nucleotides are purines, and wherein at least one PPT acts as a primer for reverse transcription.

The oligonucleotide need not match the target sequence exactly; it may span only a portion of the PPT and some mismatches may be included. The length of the oligonucleotide is preferably from about 8 to about 54 nucleotides long.  
15 The oligonucleotides should be a size which is long enough to bind specifically to the target PPT region, but not too large to prohibit entry into a cell. It will be understood that an antisense oligonucleotide may be longer than a triplex-forming  
20 oligonucleotide since the former hybridizes via Watson-Crick bonds which may extend further than the polypurine rich region while the latter forms Hoogsten-type bonds which are most efficiently formed in a polypurine rich region.

The PPT region is essential for HIV-1 replication,  
25 as demonstrated by a non-infectious RT/RNase H mutant which cannot initiate plus-strand synthesis at the PPT *in vitro*. B. Wohrl et al., *J. Mol. Biol.* 1991, 220, 801-818. Accordingly, embodiments of the invention whereby the oligonucleotide or derivative is targeted against the PPT region of HIV-1 are  
30 preferred.

The binding of the oligonucleotide to the target PPT sequence may have several related meanings. The present invention comprehends binding of an oligonucleotide or derivative with at least one portion of a PPT. It will be understood  
35 that in one embodiment of the present invention, the oligonucleotide or derivative will bind with single-stranded RNA in a Watson-Crick fashion so as to form, locally, a heteroduplex

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between the RNA and the oligonucleotide or derivative. This heteroduplex is capable of increasing premature termination of cDNA synthesis at the PPT and therefore inhibits viral replication.

5           An increase in premature termination of cDNA synthesis can be measured, for example, by incubating with reverse transcriptase for about 15 minutes the following: a viral RNA substrate, spanning the region containing the PPT and preferably about 40 to about 400 nucleotides long, the  
10   oligonucleotide to be tested, preferably 1 to 50-fold excess over the RNA substrate, and radiolabeled nucleotides. See for example, Worhl and Moelling, *Interaction of HIV-1 Ribonuclease H with Polypurine Tract Containing RNA-DNA Hybrids*, *Biochemistry*, 1990, 29, 10141-10147. At least about a 50% increase in  
15   premature termination of cDNA synthesis at the PPT, as determined by gel electrophoresis, indicates a significant increase in premature termination; a substantially complete blocking of cDNA synthesis, preferably at least about 90% and more preferably approaching 100% is preferred.

20           It will also be understood by those skilled in the art that the triplex may be formed, for example, by pairing of the purine strand to a complementary pyrimidine strand where, e.g., a C is used in the triplex-forming oligomer for every G in the purine strand and a T is used for every A, and complementary nucleotides are used for non-purines. Alternatively,  
25   a G may be used for every GC pair and a T for every AT base pair. See WO 90/06934 (Hogan, 1990). The term "triplex-forming oligonucleotide" in the present context is meant to include both of these alternatives. The binding may occur in  
30   parallel or anti-parallel orientation. The triplex results in substantial protection from RNase H where RNA-DNA hybrids are involved and likewise prevents viral replication.

          In yet another aspect of the present invention, viral replication is inhibited using a chimeric oligonucleotide of  
35   the formula 5'-A-B-C-3'. "A" comprises a segment complementary to PPT on single-stranded viral RNA, in parallel orientation. "B" comprises a linker of at least two to three, and preferably

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four, nucleotides. Preferably, the linker comprises four nucleotides. More preferably, the four nucleotides comprise thymidine. "C" comprises a segment substantially complementary to PPT, in anti-parallel orientation. Since the complementarity of segment A of the oligonucleotide facilitates binding of "A" to the PPT portion of the single-stranded RNA in parallel orientation, and the complementarity of sequence C facilitates the binding of "C" to PPT in an anti-parallel orientation, use of a linker segment--B--connecting segments A and C enables a single oligonucleotide to form a triplex with single-stranded RNA. See Figures 2a and 2b.

Heretofore, triplexes discussed in the literature were formed with double-stranded DNA. The formation of triplexes using single-stranded nucleic acids and, more specifically, between single-stranded nucleic acids and a single oligonucleotide, has not been disclosed.

The chimeric oligonucleotides of the present invention facilitate formation of "A-R-C" triplexes, with "R" representing the single-stranded RNA. Triplex formation occurs at the single-stranded RNA stage of viral reproduction. Thus, inhibition at a much earlier stage in the replication cycle is facilitated which, in turn, affords more complete inhibition at a lower concentration of oligonucleotide. Use of a single oligonucleotide which binds to single-stranded RNA also ensures greater bioavailability *in vivo* for therapeutics. Furthermore, the RNA can be targeted in the cytoplasm and at two different stages--before the RNA enters the nucleus and immediately after it exits the nucleus--increasing the potential for more effective inhibition. With prior triplex forming methods double-stranded DNA was targeted. Not only does accessibility of the DNA in the nucleus present an additional barrier for potential therapeutics, but also it is more difficult to intercalate a third strand into a pre-existing double-stranded DNA.

Segment A of the chimeric oligonucleotides of the present invention is generally at least 8, preferably 16, and more preferably 25 nucleotides long. Longer nucleotide

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segments are also contemplated. Greatest inhibition is observed when the sequence is identically complementary with at least 16 nucleotides of the PPT region. However, mismatches of between 10-20% within a region of complementarity are acceptable. The nucleotides of segment A can consist of ribonucleotides, deoxyribonucleotides, or, most preferably, ribonucleotide or deoxyribonucleotide derivatives with backbone modifications wherein the phosphodiester are replaced by phosphorothioates.

10 Segment B is generally a linker consisting of at least 2-3, and preferably four, nucleotides. In the examples which follow, a linker consisting of four thymidines was specifically utilized.

Segment C is generally at least 8, preferably 16, and most preferably 25 nucleotides long. Longer nucleotide segments are also contemplated. Segment C is substantially complementary to the PPT region in anti-parallel orientation. In the examples which follow, segment C comprises deoxyribonucleotides. Ribonucleotides are also contemplated. Again, mismatches of between 10-20% in the region of complementarity can be tolerated.

Several of the oligonucleotides tested conferred 100% protection on the single-stranded RNA. One of the oligonucleotides did not exhibit triplex formation in the tests utilized, but inhibited RNA degradation 100%. Another oligonucleotide tested formed a "pseudocircle" due to complementary tails. The pseudocircle conferred 80% protection against RNA degradation. The oligonucleotides were added in micro-nanomolar amounts.

Substantial protection against RNase H degradation can be measured, for example, by incubating at 37°C overnight the following: a radiolabeled RNA substrate, preferably about 100 to 400 nucleotides long, a DNA substrate, preferably about 8 to about 40 nucleotides long, both of which include the PPT, and the antisense oligonucleotide to be tested, in an amount which is preferably about 2 to about 100-fold in excess of the RNA substrate. The mixture is then incubated for about 30 minutes at 37°C with RNase H in RNase H buffer with a substrate

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to enzyme ratio of about 4 to 1. See, for example, Worhl and Moelling, *Interaction of HIV-1 Ribonuclease H with Polypurine Tract Containing RNA-DNA Hybrids*, *Biochemistry*, 1990, 29, 10141-10147 and Wohrl et al., *J. Mol. Biol.* 1991, 220, 801-818.

5 It is known that RNase H preferentially cleaves at two sites within the PPT, at nucleotides 72 and 79. Worhl and Moelling, *Biochemistry*, 1990, 29, 10141-10147. Substantial protection against RNase H digestion is measured by the reduction of at least one cleavage product by at least about  
10 50% and preferably at least about 80%, as determined by gel electrophoresis and using a control lane of the same reactants with RNase H. This measurement can be made, for example, by densitometric scanning.

The same oligonucleotide or derivative targeted  
15 against a PPT present in RNA may also be capable of forming a triplex with the PPT in a double-stranded DNA copy, thus protecting the PPT region in double-stranded DNA from DNase I digestion. However, such cannot be assumed due to chemical and structural differences between RNA and DNA. A triplex with  
20 double-stranded DNA would presumably prevent viral transcription since it occurs at a stage where the virus has already replicated its RNA genome into a double-stranded DNA copy. The double-stranded DNA stage is thus unlike the single-stranded RNA and RNA-DNA hybrid stages above which may arise before  
25 viral replication is complete. Intervention at the earlier stage is preferable since it should confer an advantage in combatting infection by inhibiting viral replication. Further, the targets are present in the cytoplasm, not in the nucleus.

The oligonucleotides may be synthetic deoxyribonucle-  
30 otides, ribonucleotides, combinations (chimeras), or derivatives thereof. The use of ribonucleotide derivatives has been reported by Shibahara et al., *Nucleic Acids Research* 1989, 17, 239-252. A person skilled in the art is capable of synthesizing oligonucleotides. See for example, Gait, M.J., ed. (1984)  
35 *Oligonucleotide Synthesis* (IRL, Oxford).

Derivatives contemplated include modifications of the nucleotides which increase bioavailability by enhancing

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stability to nuclease attack and/or increasing cellular uptake. For example, the modification of the nucleotide backbone bonds from phosphodiester to phosphorothioates confers greater stability against nuclease attack. Phosphorothioates, i.e.,  
5 the substitution of a sulfur atom for a phosphate oxygen in the internucleotide phosphodiester linkage, are stable to nuclease cleavage and soluble in lipid. Lipid solubility can be important for bioavailability, as discussed further below regarding liposomes. Preferably, only the two terminal bonds  
10 at either, or both, ends of the oligonucleotides are modified. In this manner, stability is increased without foregoing cellular uptake. The oligonucleotides can also be protected from exonuclease attack through the addition of amino groups at the ends. Derivatives such as methylphosphonates,  
15 phosphotriesters, phosphorothioates and phosphoroamidates are also contemplated. Additionally, beta-anomers may be replaced with alpha-anomers. Sun et al., *Triple-Helix Formation by  $\alpha$  Oligodeoxynucleotides and  $\alpha$  Oligodeoxynucleotide-Intercalator Conjugates*, *Proc. Natl. Acad. Sci.* 1991, 88, 6023-6027. See  
20 also C.A. Stein & J.S. Cohen, *Oligodeoxynucleotides as Inhibitors of Gene Expression: A Review*, *Cancer Research* 1988, 48, 2659-2668.

While any length oligonucleotide may be utilized, sequences shorter than 15 bases may be less specific in  
25 hybridizing to the target and may be more easily destroyed by enzymatic degradation. The chimeric oligonucleotides of the present invention having the formula 5'-A-B-C-3' consist of at least 20 nucleotides or more. Conversely, the size of the oligonucleotide may limit its ability to enter the target cell.  
30 Large oligonucleotides may be somewhat less effective in interfering with expression because of decreased uptake by the target cell. However, carriers can be employed to further increase the uptake of the oligonucleotides.

It is generally preferred to administer the oligo-  
35 nucleotides and their derivatives to an infected individual in accordance with this invention internally, such as orally, intravenously or intramuscularly. Other forms of administra-

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tion, such as transdermally, topically, or intralesionally may also be useful. Inclusion in suppositories can also be useful. Use of the oligonucleotides and their derivatives in prophylaxis is also likely to be useful. Use of pharmaceutically acceptable carriers is also preferred for some embodiments.

Pharmaceutical compositions of this invention comprise a pharmaceutically acceptable carrier or diluent and an effective quantity of one or more of the oligonucleotides or their derivatives, or an acid or base salt thereof. The carrier or diluent may take a wide variety of forms depending on the form of preparation desired for administration, e.g., sublingual, rectal, nasal, oral, or parenteral.

In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, for example, waters, oils, alcohols, flavoring agents, preservatives, and coloring agents, to make an oral liquid preparation (e.g., suspension, elixir, or solution) or with carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, and disintegrating agents, to make an oral solid preparation (e.g., powder, capsule, or tablet).

Controlled release forms or enhancers to increase bioavailability may also be used. Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are employed. If desired, tablets may be sugar coated or enteric coated by standard techniques.

For parenteral products, the carrier will usually be sterile water, although other ingredients to aid solubility or as preservatives may be included. Injectable suspensions may also be prepared, in which case appropriate liquid carriers and suspending agents can be employed.

The oligonucleotides and their derivatives can also be administered locally at a lesion by topical application of a solution or cream.

Alternatively, the oligonucleotides or their derivatives may be administered in liposomes or microspheres (or microparticles). Incorporation of the oligonucleotides into



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liposomes can improve the bioavailability of the oligonucleotides for therapy. Furthermore, incorporation of the oligonucleotides into immunoliposomes, such as discussed in Uhlmann, et al., "Antisense Oligonucleotides: A New Therapeutic Principles", *Chemical Reviews*, Volume 90, No. 4, pages 544-584, June 1990 (incorporated herein by reference) can help to specifically target the oligonucleotides. Methods for preparing liposomes and microspheres for administration to a patient are known to those skilled in the art. U.S. Patent No. 4,789,734 describes methods for encapsulating biological materials in liposomes. Essentially, the material is dissolved in an aqueous solution, the appropriate phospholipids and lipids added, along with surfactants if required, and the material dialyzed or sonicated, as necessary. A review of known methods is provided by G. Gregoriadis, Chapter 14, "Liposomes", *Drug Carriers in Biology and Medicine*, pp. 287-341 (Academic Press, 1979). Microspheres formed of polymers are well known to those skilled in the art, and can be tailored for passage through the gastrointestinal tract directly into the bloodstream. Alternatively, the nucleotides or their derivatives can be incorporated and the microspheres, or composite of microspheres, implanted for slow release over a period of time, ranging from days to months. See, for example, U.S. Patents Nos. 4,906,474, 4,925,673 and 3,625,214.

Tests show that the best results are achieved when the oligonucleotides are administered in an excess of at least about 2-fold, preferably at least about 10-fold, to the target single-stranded RNA or RNA-DNA hybrid. An optimal dosage for an infected individual will, of course, depend on the size of the individual and the type and extent of infection. Generally, however, the oligonucleotides or their derivatives should be active when administered parenterally in amounts above about .1 to about 30 mg/kg body weight. Effective doses by other routes of administration should generally be those which result in similar blood level to i.v. doses above about .1 to about 30 mg/kg. Uhlmann et al. reported that multiple administration of small amounts of oligonucleotide appeared to have a

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more beneficial effect than a single large dose. Anfossi, et al., *Proc. Natl. Acad. Sci. USA*, 1989, 3379-3383 reported the application of oligonucleotides to cell culture in three doses with an inhibitory effect on proliferation of human myeloid  
5 leukemia cell lines. Others reported positive results *in vivo* in mice using a regimen of oligonucleotide dispensed continually over a period of 7-14 days. Ratajczak, et al., *Proc. Natl. Acad. Sci. USA*, December 1992, 89, 11823-11827.

In Ratajczak et al., phosphorothioate-modified  
10 antisense oligonucleotides against the *c-myc* protooncogene were utilized. Mice received a total of 5 mg/kg body weight oligonucleotide per day over a dosage period of 7-14 days. Doses were administered at 1 microliter per hour, subcutaneously into a paraspinal pocket. Leukemic mice receiving the  
15 therapy lived 3.5 times longer than leukemic mice not receiving the therapy. Mice were induced with human leukemia.

The criteria for assessing response to therapeutic modalities employing these nucleotides, and, hence, effective dosages of the nucleotides of this invention for treatment, are  
20 dictated by the specific condition and will generally follow standard medical practices. Overall, it is preferred to administer to patients suspected of suffering from RNA virus disease states with effective amounts of oligonucleotides or derivatives, in either native form or suspended in a carrier  
25 medium in amounts and upon treatment schedules which are effective to reduce the symptomology of disease. It is within the scope of a person's skill in the art to determine optimum dosages and treatment schedules for such treatment regimens.

The following non-limiting examples are meant to  
30 illustrate several embodiments of the invention.

## EXAMPLES

### EXPERIMENTAL PROCEDURES

**Materials.** Restriction enzymes, bacterial alkaline phosphatase, and the vanadyl ribonucleoside complex (VRC) were  
35 purchased from BRL, Berlin, West Germany. *Hae*III-restricted pBR322 DNA, T4 polynucleotide kinase, T4 DNA ligase, RNase-free

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DNase I, *E. coli* RNase H, 2'3'-dideoxynucleoside triphosphates (dNTPs) were obtained from Boehringer Mannheim, Mannheim, Best Germany. RNasin was purchased from Promega Biotech, Heidelberg, West Germany. Sequencing reactions were performed with the Sequenase kit from USB, Cleveland, OH. AMV RT and the vector pTZ19R harboring the T7 promoter were obtained from Pharmacia, Uppsala, Sweden. Radiolabeled compounds [ $\gamma$ - $^{32}$ P] ATP (3000 Ci/mmol), [ $\alpha$ - $^{32}$ P]UTP (.400 Ci/mmol), [ $\alpha$ - $^{32}$ S]dATP $\alpha$ S (>1000 Ci/mmol), and [ $^3$ H]TTP (42 Ci/mmol) were purchased from Amersham Buchler, Braunschweig, West Germany. The RNA transcription kit, T7 RNA polymerase, and AMV reverse transcriptase sequencing kit were purchased from Stratagene, Heidelberg, West Germany. DNA oligonucleotides were synthesized on an Applied Biosystems oligonucleotide synthesizer and were purified by HPLC prior to use.

*DNA Manipulations.* Restriction endonuclease cleavage, DNA isolation, ligation, end labeling of DNA, and transformations were performed as described in Maniatis et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, NY 1982).

*Plasmid Constructions.* The 474 base pair long PvuII fragment from the HIV-1 provirus clone BH10 (supplied by Dr. R.C. Gallo) including the polypurine tract (PPT) was cloned into the SmaI site of the T7 transcription vector PTZ19R to yield plasmid pTZp8, which was further used in RNA transcription assays. The same PvuII fragment was cloned in both orientations into DNA of the phage M13mp18. For isolation of DNA, the constructs were transformed into the *E. coli* K12 strain JM109 according to Yannisch-Perron et al., *Gene*, 1985, 33, 103-119. Plasmids pKJ2 and pKJ11 were derived from pTZp8 as indicated in Figure 1.

*In Vitro Transcription with Plasmids pTZp8, pKJ2 and pKJ11.* A total of 1  $\mu$ g of DNA of plasmid pTZp8, pKJ2 or pKJ11 was linearized with EcoRI and, after proteinase K treatment and phenol/chloroform extraction, was transcribed by T7 RNA

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polymerase according to the conditions described in the Stratagene RNA transcription kit. Thus, an RNA of 534 bases in length was synthesized including the PPT and flanking regions where pTZp8 was used and an RNA of 213 or 134 bases where pKJ2 or pKJ11 was used (see Figure 1). High specific RNA probes were uniformly labeled with 40  $\mu$ M UTP and 50  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]UTP, and low specific RNA was obtained with 200  $\mu$ M UTP and 50  $\mu$ Ci of [ $\alpha$ -<sup>32</sup>P]UTP.

*Purification of HIV-1 RT/RNase H.* The bacterially expressed recombinant RT/RNase H was purified as described previously with a poly(U)-Sepharose instead of an Aga(rC) column according to Hansen et al., *J. Biol. Chem.* 1987, 262, 12393-12396 and Hansen et al., *EMBO J.* 1988, 7, 239-243. One unit of purified HIV-1 RT/RNase H catalyzes the incorporation of 13 pmol of [<sup>3</sup>H]TMP into acid-insoluble products in 10 minutes at 37°C with poly(Ra)•(dT)<sub>10</sub> as template/primer. The concentration of the enzyme corresponds to about 1  $\mu$ L per unit.

*Sequencing Reactions.* Sequencing reactions were carried out by using the dideoxy sequencing kit from USB according to the manufacturer's instructions except with a <sup>32</sup>P-end-labeled oligonucleotide primer in some reactions. Aliquots were analyzed on 8% or 10% TBE-urea-polyacrylamide gels.

#### Example 1 SINGLE-STRANDED RNA

HIV-1 viral RNA was transcribed *in vitro* from a construct containing a T7 promoter along with the PPT, which consists of the sequence: AAAAGAAAAGGGGGGA (SEQ ID NO: 1) or AAAAGAAAAGGGGGGACTGGAAGGGC (SEQ ID NO: 2) when extended within the 3' LTR and 2 non-purines are included. The viral RNA, corresponding to pKJ2, consisted of 134 nucleotides. A <sup>32</sup>P end-labeled antisense oligonucleotide was hybridized with the RNA. The cDNA reaction products were analyzed on 10% polyacrylamide TBE-urea gels. Details of the procedure are as follows:

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*RNase H Reaction Coupled with cDNA Elongation.* To the standard reaction mixture described in Example 2 below were added a 125  $\mu$ M quantity of one of the ddNTPs and each of the other three dNTPs and 4 units of HIV-1RT. The reactions were started by the addition of approximately 3 pmol of hybrid RNA (low specific activity) and treated as described above. To check for cDNA elongation, the same test was carried out, except with unlabeled *in vitro* transcribed RNA hybridized to a 10:1 mixture of unlabeled to labeled oligonucleotide. Aliquots were analyzed by electrophoresis on 10% TBE-urea-polyacrylamide gels.

*RNase H Reaction and Oligonucleotide Extension Assays of RNase H Treated Model Substrates X and Y.* The RNase H reaction was carried out in a total volume of 500  $\mu$ L in RNase H buffer containing 1000 units of RNasin and 25 units of HIV-1RT/RNase H. After the reaction was started by the addition of 8 pmol of an unlabeled RNA-DNA oligonucleotide hybrid, the sample was divided into five aliquots of 100  $\mu$ L and incubated for 30 minutes at 37°C, and the reaction was stopped at 95°C for 5 minutes. The samples were placed on ice and to each was added 10 units of DNase I to remove the DNA oligonucleotide. After 10 minutes, the samples were incubated further at 37°C for 10 minutes. The reactions were stopped at 95°C for 10 minutes. A  $^{32}$ P-end-labeled DNA oligonucleotide primer N complementary to a sequence 66-86 bases downstream of the PPT was annealed to the cleaved RNA and the primer extended up to the RNase H cleavage site in a total volume of 15  $\mu$ L in 1 X RT buffer (Stratagene sequencing kit) by means of 7 units of AMV RT (Stratagene sequencing kit), 20 units of RNasin, and dNTPs to 330  $\mu$ M. After 1 hour at 42°C, the reaction was stopped with 5  $\mu$ L of formamide buffer included in the kit. Aliquots were analyzed by electrophoresis on an 8% TBE-urea-polyacrylamide gel together with dideoxy sequencing ladders generated with the same  $^{32}$ P-end-labeled DNA oligonucleotide primer on a single-stranded M13 DNA template harboring the corresponding DNA fragment.

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*RNase H Reaction and Reverse Transcription Using an M13 RNA Hybrid as Substrate.* A total of 2.5  $\mu\text{g}$  of *in vitro* transcribed RNA and 8  $\mu\text{g}$  of the M13 single-stranded DNA harboring the complementary fragment were hybridized in RNase H buffer in a total volume of 25  $\mu\text{L}$  by heating the mixture to 80°C and cooling it slowly (1-2 hours) to room temperature. The RT/RNase H reaction was carried out in a total volume of 50  $\mu\text{L}$  in RNase H buffer containing 50 units of RNasin, 10 units of HIV-1 RT/RNase H, and a 150  $\mu\text{M}$  quantity of each dNTP. The reaction was started by the addition of 25  $\mu\text{L}$  of the hybridization mixture, the mixture was incubated for 40 minutes at 37°C, and the reaction was terminated by heating the sample to 95°C for 3 minutes. After treatment with 0.3 M NaOH at 65°C for 20 minutes, ammonium acetate (pH 4.8) was added to 3 M, and the products were precipitated with 4 volumes of absolute ethanol and washed three times with 70% ethanol to remove salt and free nucleotides. An oligonucleotide extension assay was carried out with the  $^{32}\text{P}$ -end-labeled DNA oligonucleotide N described above in 1 X Sequenase buffer (Sequenase Kit) in a total volume of 20  $\mu\text{L}$ . The mixture contained 5 mM DTT, a 250  $\mu\text{M}$  quantity of each dNTP, and 7.5 units of Sequenase and was incubated for 40 minutes at 37°C. The reaction was stopped by the addition of 5  $\mu\text{L}$  of formamide buffer included in the Sequenase kit. Electrophoresis was performed on a 10% gel along with dideoxy sequencing ladders generated on the corresponding M13 DNA template with the unlabeled DNA primer N and [ $\alpha$ - $^{35}\text{S}$ ]dATP $\alpha$ S. See Wohrl and Moelling, *Interaction of HIV-1 Ribonuclease H with Polypurine Tract Containing RNA-DNA Hybrids*, *Biochemistry* 1990, 29, 10141-10147 and B. Wohrl et al., *Mutations of a Conserved Residue within HIV-1 Ribonuclease H Affect Its Exo- and Endonuclease Activities*, *J. Mol. Biol.* 1991, 220, 801-818.

The results indicated that premature termination of cDNA synthesis at homopolymeric stretches of the RNA template is most prominent at the PPT. This natural premature termination can be further increased by the addition of an antisense oligonucleotide which binds to the PPT region. An antisense 20-mer, AGTCCCCCTTTTCTTTTAA (SEQ ID NO: 10) and an antisense

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40-mer, TGAATTAGCCCTTCCAGTCCCCCCTTTTCTTTTAAAAAGT (SEQ ID NO: 9), both of which cover the PPT, were used and led to complete (100%) blocking of cDNA synthesis with two to three prominent stops. An antisense 20-mer outside the PPT region,  
 5 CCGCCCAGGCCACGCCTCCC (SEQ ID NO: 11), was much less inhibitory (20-50%) for cDNA elongation than the 20-mer covering the PPT (100%).

TABLE 2

Y (40-mer) = 58-97: (SEQ ID NO: 9)  
 10 TGAATTAGCCCTTCCAGTCCCCCCTTTTCTTTTAAAAAGT

X (20-mer) = 63-82: (SEQ ID NO: 10)  
 AGTCCCCCCTTTTCTTTTAA

CONTROL = S (20-mer) = 465-484: (SEQ ID NO: 11)  
 CCGCCCAGGCCACGCCTCCC

15 **Example 2 pJK11 RNA-DNA HYBRIDS**

Viral RNA was transcribed *in vitro* from a construct containing a T7 promoter along with the PPT, and the RNA was radiolabeled. The viral RNA, corresponding to pKJ11, consisted of 213 nucleotides. A DNA oligomer consisting of 40 oligo-  
 20 nucleotides, TGAATTAGCCCTTCCAGTCCCCCCTTTTCTTTTAAAAAGT (SEQ ID NO: 9), spanning the PPT region was synthesized *in vitro* and was utilized as a strand in the RNA-DNA hybrid. Two 25-mers, TFO 1 and TFO 1' and two 16-mers, TFO 4 and TFO 4', the triplex-forming oligonucleotides (TFOs), were also synthesized  
 25 *in vitro*. The TFO's were added at 10-Fold and 100-Fold molar excess.

TABLE 3

TFO 1 25-mer parallel polypyrimidine: (SEQ ID NO: 3)  
 TTTTCTTTTCCCCCTGACCTTCCC

30 TFO 1' 25-mer parallel mixed purines & pyrimidines: (SEQ ID NO: 4)

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TTTTGTTTTGGGGGGTGTGGTTGGG

TFO 4 16-mer parallel polypyrimidine: (SEQ ID NO: 5)

TTTTCTTTTCCCCCCT

TFO 4' 16-mer parallel mixed purines &amp; pyrimidines: (SEQ ID NO:

5 6)

TTTTGTTTTGGGGGGT

The same RNA-DNA hybrid substrate but without a TFO was used as a control.

Triplex formation was performed under physiological conditions using 10 mM magnesium, 80 mM sodium chloride, spermidine, and a pH of 7.2. RNA and DNA were preheated to 96°C and allowed to cool slowly. The preheating in this and subsequent examples was to prevent the formation of secondary structures by the "naked" nucleic acids *in vitro* which may inhibit binding. *In vivo*, melting proteins, such as histones, prevent the formation of interfering secondary structure. The incubation was performed overnight at 37°C to allow triplex formation. In general, RNase H cleavage of RNA-DNA oligonucleotide hybrids was measured as follows:

Standard reactions were carried out in a total volume of 20  $\mu$ L in RNase H buffer (50 mM Tris-HCl, pH 7.8, 40 mM NaCl, 1 mM MgCl<sub>2</sub>, 2 mM DTT) containing 20 units of RNasin. A total of 0.2 unit of HIV-1 RT/RNase H, 3 units of AMV RT/RNase H, or 4 units of RNase H from *E. coli* was added. (Unit definitions of AMV RT and RNase H from *E. coli* are according to the descriptions given by the manufacturers.) Reactions were started by the addition of 0.2 pmol of the RNA (high specific activity) prehybridized in the presence of a 20-25-fold molar excess of the corresponding DNA oligonucleotide. The mixture was incubated for 30 minutes at 37°C, and the reactions were terminated by the addition of 6  $\mu$ L of urea loading buffer (7 M urea in 1 X TBE, 0.1% each xylene cyanol and bromophenol blue). Aliquots were analyzed by electrophoresis on a 10% TBE-



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urea-polyacrylamide gel. See also Maher et al., *Biochemistry* 1990, 29, 8820-8826 for details of the procedure.

Two concentrations of the TFO were used; 10- and 100-fold excess compared to the hybrid and the TFO's were incubated  
5 with recombinant RT/RNase H.

All of the TFO's shown in Table 3 led to substantial protection against RNase H digestion. Nonetheless, a 25-mer, sequence, TFO 1', was two- to four-fold more efficient than a 16-mer, TFO 4', at concentrations of both 10- and 100-fold  
10 excess. TFO 4' led to some effect, albeit two-fold less efficient, at 10-fold molar excess. The TFO's which had a G for every G-T pair and a T for every A-T pair (TFO 1' and TFO 4') had greater efficiency than the polypyrimidine TFO's (TFO 1 and TFO 4).

15 A 213-nucleotide long RNA corresponding to pKJ11 was end-labeled with <sup>32</sup>P in vitro. This RNA includes the PPT. The RNA was mixed with the PPT-covering DNA oligonucleotide in a ratio of 1 to 2 or 1 to 10, heated to 100°C and allowed to slowly cool. The mixture was incubated with the TFO oligonu-  
20 cleotide at 37°C overnight. Three different TFO's were used, designated TFO 1, TFO 2 and TFO 3.

TABLE 4

TFO 1 25-mer parallel polypyrimidine: (SEQ ID NO: 3)

TTTTCTTTTCCCCCTGACCTTCCC

25 TFO 2 25-mer parallel polypurine: (SEQ ID NO: 7)

AAAAGAAAAGGGGGGACTGGAAGGG

TFO 3 25-mer anti-parallel polypyrimidine: (SEQ ID NO: 8)

CCCTTCCAGTCCCCCTTTTCTTTT

Incubation with purified recombinant RNase H was  
30 performed under standard assay conditions as published in Wohrl, B et al., *Mutations of a Conserved Residue within HIV-1 Ribonuclease H Affect Its Exo- and Endonuclease Activities*, *J. Mol. Biol.* 1991, 220, 801-818. Triplex formation was performed under physiological conditions using 10 mM magnesium, 80 mM  
35 sodium chloride, spermidine, and a pH of 7.2. RNA and DNA were

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preheated to 96°C and allowed to cool slowly. The incubation was performed overnight at 37°C to allow triplex formation. The incubation period with RNase H was performed at 37°C for 15 minutes. See Example 2 above and Maher et al., *Biochemistry* 5 1990, 29, 8820-8826 for details of the procedure.

All TFO's in Table 4 showed substantial RNase H protection when compared to the control, wherein the hybrid substrate without a TFO was incubated with RNase H, but TFO's 2 & 3 showed greater protection. Protection against RNase H 10 digestion indicates the formation of a triplex which would be expected to interfere with viral replication *in vivo*, given the results obtained with RT/RNase H mutants.

### EXAMPLE 3 pJK2 RNA-DNA HYBRIDS

*In vitro* transcribed, 5'-end-labeled pKJ2 RNA of 134 15 nucleotides in length was hybridized with a 10-fold excess of a 40-mer deoxyribonucleotide complementary to the PPT region. Volkmann, et al., *Biochimie*, 1993, 75, 71-78. Hybridizations were carried out in 25 mM Tris-acetate buffer (pH 6.8) containing 50 mM NaCl, 10 mM  $\beta$ -mercapotoethanol and 0.4 mM spermine 20 hydrochloride. The mixture was incubated for 3 minutes at 90°C, then cooled slowly to room temperature. For triplex-formation, 1 pmol hybrid substrate was incubated with either 10 or 100 pmol oligodeoxynucleotide overnight at 37°C. Finally, each sample was treated with 12 ng enzyme in 10 25 microliters RNase H-standard buffer (see Example 2) for 30 minutes at 37°C. RNase H cleavage reaction was stopped by incubation at 96°C for 2 minutes. Samples were ethanol-precipitated and analyzed on 10 % polyacrylamide-TBE-urea gel. The gel was visualized by autoradiography and analyzed with a 30 phosphoimager (Molecular Dynamics). GT purine-pyrimidine mixed DNA oligonucleotides and RNA oligonucleotides were analyzed and compared with a 25-mer pyrimidine sequence in parallel (Seq ID No: 3) and antiparallel (Seq ID No: 42) orientation. To 35 quantitate the effects of the oligonucleotides, the amounts of uncut RNA were determined. The oligonucleotides tested are listed below:

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TFO 1    TTTTCTTTTCCCCCTGACCTTCCC    (SEQ ID NO:3)  
 (PY1)  
 PY2       CCCTTCCAGTCCCCCCTTTTCTTTT    (SEQ ID NO:42)  
 (TFO 1)  
 5    TFO 1'    TTTTGTTTTGGGGGGTGTGGTTGGG    (SEQ ID NO:4)  
 (GT1)  
 GT2       GGGTTGGTGTGGGGGGTTTTGTTTT    (SEQ ID NO:39)  
 GT3       TGGGGGGTTTTGTTTT    (SEQ ID NO:40)  
 10    TFO 4'    TTTTGTTTTGGGGGGT    (SEQ ID NO: 6)  
 (GT 4)  
 GU1       UUUUGUUUUGGGGGGUGUGGUUGG    (SEQ ID NO:41).

As is evident from the above, GU1 was the RNA oligonucleotide tested.

15            All oligonucleotides tested inhibited RNase H to some degree. The "PY2" pyrimidine sequence (SEQ ID NO:42), the antiparallel of TFO 1, exhibited the lowest degree of inhibition. Oligonucleotide GU1 approached 100% inhibition.

Oligonucleotides GT1, GT2, and GU1 were tested  
 20 further at a range of concentrations. The concentration ranges are indicated in Figure 4. The concentrations of the remaining materials were as previously. The quantitative plots of phosphoimager results for GT1, GT2, and GU1 are depicted in Figure 4. GT1 inhibited the RNase H by 50 % at 5.0  $\mu$ M, and  
 25 GU1 inhibited 50 % at 0.5  $\mu$ M. Since GT1 forms a DNA-RNA-DNA triplex, and GU1 forms a DNA-RNA-RNA triplex, these results indicate that the DNA-RNA-RNA triplex is more protecting.

#### **EXAMPLE 4    SINGLE-STRANDED RNA TRIPLEXES**

Chimeric oligonucleotides of the basic formula 5'-A-  
 30 B-C-3' were tested with single-stranded RNA in a two-strand system. Triplexes were allowed to form *in vitro* as in Example 3 above, with the exception that the pH was adjusted to 6.5 to comport with what is believed to be the local pH of triplexes which form naturally *in vivo*. (Personal communication.)  
 35    Average protection from cleavage was about 50%.

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Protection from RNase H was monitored as above. Specifically, protection was monitored *in vitro* using RNA which was radioactively labeled at its 5'-end. Cleavage was analyzed on a sequencing gel. Cleavage within the PPT region of the RNA  
5 leads to a band of about 80 nucleotides. Protection from cleavage results in an uncleaved full-length RNA. The amount of cleaved versus uncleaved RNA was monitored by phosphorimager.

The oligonucleotide of the formula 5'-A-B-C-3' was synthesized with segment A comprising ribonucleotides and  
10 deoxyribonucleotides, alternatively. Segment C was synthesized using deoxyribonucleotides. Ribonucleotides can also be used for segment C. However, if segment A is synthesized using ribonucleotides, it is preferable that segment C be synthesized using deoxyribonucleotides. Greatest protection was achieved  
15 with segment A comprising 16 ribonucleotides and segment C comprising 16 deoxyribonucleotides, linked by four thymidines. One hundred percent protection from cleavage was achieved with a phosphorothioate-modified oligonucleotide comprising this structure (SEQ. ID No: 37) at equimolar concentration.

20 The formation of a triplex at the PPT was confirmed using a primer-extension technique. A primer binding downstream of the PPT RNA was synthesized and extended *in vitro* by RT in the presence of oligodeoxynucleotides, including one which was radioactively labelled. The newly synthesized DNA  
25 was terminated at the site of the PPT when the triplex was formed and blocked extension. Additionally, a melting profile was performed whereby measurements of optical absorption revealing two transition points proved that a triplex had formed.

30 The chimeric oligonucleotides tested are depicted in Tables 5 through 7. Test designations and triplex conformations are indicated in the first column. Ribonucleotides are indicated in Table 7 with a subscript "r". Also in Table 7, phosphorothioate derivatives of the nucleotides are indicated  
35 with a subscript "s". The linker segments are underlined in all tables. All oligonucleotides exhibited formation of a

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triplex by the test methods used, with the exception of the oligonucleotide labeled TFO A in Table 5 (SEQ. ID No:12).

TFO A is 54-mer which is partially self-complementary and forms a hairpin-type stem-looped structure containing two mismatched regions, a loop and loose ends. See Figures 3a and 3b. TFO A completely inhibited cleavage at concentrations less than one nanomolar, *in vitro*. TFO A takes a structural conformation which resembles that of the PPT and, due to its self-complementarity, is very stable. Thus, it is possible that TFO A's inhibitory effect is strong because it mimics the PPT hybrid region.

TFO A (SEQ ID No:12) was modified through nucleotide changes as reported in Table 6. Through these studies, it was learned that the two bulges are essential for protection of RNA and that the overhanging 5'- and 3'-ends can be shortened. Variances numbered 5037 (SEQ ID NO:26) and 5074 (SEQ ID NO:21) inhibited cleavage to 50% at 50-fold molar excess of the oligonucleotide over the template, i.e., about one  $\mu\text{M}$  oligonucleotide. Ninety-five percent inhibition of the RT occurred at about 25  $\mu\text{M}$ .

Derivatives of oligonucleotide TFO F tested are presented in Table 7. The phosphorothioate derivative of SEQ ID No:37 ( $R_sRD$  in Table 7) resulted in 100% inhibition at the same concentration as the phosphodiester, thereby exhibiting an increase in protection over the phosphodiester form. Derivative TFO  $F_1$  (SEQ ID No:38) formed a pseudocircle due to the synthesis of a complementary tail. TFO  $F_1$  inhibited RNase H by about 80%. The formation of a pseudocircle may provide increased protection from exonuclease attack.

The foregoing examples are meant to illustrate the invention and not to limit it in any way. Those skilled in the art will recognize that modifications can be made which are within the spirit and scope of the invention as indicated in the appended claims.

TABLE 5  
TFO A through F

<u>DESIGNATION</u>	
TFO A	5' TTTTCTTTTG GGGGGTTTGG TTGGGTTTTC CCTTCCAGTC CCCCCCTTTT TTTT 3' (SEQ ID NO:12)
5 TFO B	5' TGTGTGTGTG TGTGTGTGTG TGTGTGTGT CCTTCCAGT CCCCCCTTTT CTTT <sup>1</sup> 3' (SEQ ID NO:13)
TFO C	5' TTTTATTTTA GGGGATTTGG TTGGGTTTTC CCTTCCAGTC CCCCCCTTTT TTTT 3' (SEQ ID NO:14)
TFO D	5' TTTTCTTTTG GGGGGTCTGG TTGGG <sup>2</sup> TTTTCCCTTC CAGTCCCCCC TTTTCTTTT 3' (SEQ ID NO:15)
TFO E	5' TTTTGTTTTG GGGGGT <sup>3</sup> TTTTCCCCCC TTTTCTTTT 3' (SEQ ID No:16)
TFO F	5' TTTTCTTTTC CCCCCCTTTT TCCCCCCTTT TCTTTT (SEQ ID No:17)

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- 
- 10 <sup>1</sup> Portion in bold corresponds to SEQ ID No:8.  
<sup>2</sup> Portion in bold corresponds to SEQ ID No:4.  
<sup>3</sup> Portion in bold corresponds to SEQ ID No:6.

TABLE 6  
TFO-A Variants

DESIGNATION

4-5-7-2	5'	GGG GGG TTT GGT TGG GTT <u>TTC</u> CCA ACC AAA CCC CCC 3'	(SEQ ID NO:18)
5 4-5-7-1	5'	GGG GGG TTT GGT TGG GTT <u>TCC</u> CTT CCA GTC CCC CC 3'	(SEQ ID NO:19)
4-5-7-4	5'	GGG GGG TTT GGT TGG GTT <u>TTT TCC</u> CTT CCA GTC CCC 3'	(SEQ ID NO:20)
5-0-7-4	5'	AGG GGA TTT GGT TGG GTT <u>TTC</u> CCT TCC AGT CCC CC 3'	(SEQ ID NO:21)
4-5-7-3	5'	GGG GGG TTT GGT TGG GTT <u>TTT CCC</u> TTC CAG TCC CCC 3'	(SEQ ID NO:22)
5-1-5-9	5'	CCC CCT CTC TTC <u>AAA AGA</u> GAA GGG GGG 3'	(SEQ ID NO:23)
10 5-1-6-0	5'	CCC CCT TCT TCA <u>AAA GAG</u> AGG TTG GGG G 3'	(SEQ ID NO:24)
5-0-7-2	5'	GGG GAC TGG GAG GGT <u>TTT CCC</u> TTC CAG TCC CCC 3'	(SEQ ID NO:25)
5-0-3-7	5'	TTT TCT TTT GGG GGG TTT GGT TGG GTT <u>TTC CCT</u> TCC AGT CCC CCC TTT TCT TTT	
		3' (SEQ ID NO:26)	

TABLE 6 (cont.)  
TFO-A Variants

DESIGNATION

	RH 1001	5'	GGG GGG TTT GGT TGG GTT TTC CCT TCC AGT CCC CCC	(SEQ ID NO:27)
5	RH 1002	5'	GGG GGG ACT GGT TGG GTT TTC CCT TCC AGT CCC CCC	(SEQ ID NO:28)
	RH 1003	5'	GGG GGG TTG TCC CCC C 3' (SEQ ID NO:29)	
	RH 1004	5'	GGG GGG TTT GGA AGG GTT TTC CCT TCC AGT CCC CCC	(SEQ ID NO:30)
	RH 1005	5'	GGG GGG ACT GGA AGG GTT TTC CCT TCC AGT CCC CCC	(SEQ ID NO:31)
	RH 1006	5'	TTT GGT TGG GTT TTC CCT TCC AGT 3' (SEQ ID NO:32)	
10	RH 1007	5'	GGG GGG NNN GGT TGG GTT TTC CCT TCC AGT CCC CCC	(SEQ ID NO:33)
	RH 1008	5'	GGG GGG TTT GGN NGG GTT TTC CCT TCC AGT CCC CCC	(SEQ ID NO:34)
	RH 1009	5'	GGG GGG TTT GGT TGN NTT TTC CCT TCC AG CCC CCC 3' (SEQ ID NO:35)	
	RH 1010	5'	GGG GGG TTT GGT TGG GTT TTC CCT TCC AGT CCC CCC TTT TCT TTT 3'	
			(SEQ ID NO:36)	



TABLE 7  
TFO-F Derivatives

DESIGNATION/  
TRIPLEX

5	TFO F/DRD	5' TTT TCT TTT CCC CCC <u>TTT TTT</u> CCC CCC TTT TCT TTT 3' (SEQ ID NO:17)
	DR1/RRD	5' UUU UC <sub>1</sub> U UUU C <sub>1</sub> C <sub>1</sub> C <sub>1</sub> C <sub>1</sub> C <sub>1</sub> C <sub>1</sub> <u>UTT TT</u> TCC CCC CTT TTC TTT T 3' (SEQ ID NO:37)
	DR2/RsRD	5' U <sub>8</sub> U <sub>8</sub> U <sub>8</sub> C <sub>8</sub> U <sub>8</sub> U <sub>8</sub> U <sub>8</sub> C <sub>8</sub> C <sub>8</sub> C <sub>8</sub> C <sub>8</sub> C <sub>8</sub> C <sub>8</sub> <u>U<sub>8</sub>TT TT</u> TCC CCC CTT TTC TTT T 3' (SEQ ID NO:37)
	Thio/DsRD	5' T <sub>8</sub> T <sub>8</sub> T <sub>8</sub> T <sub>8</sub> C <sub>8</sub> T <sub>8</sub> T <sub>8</sub> T <sub>8</sub> T <sub>8</sub> C <sub>8</sub> C <sub>8</sub> C <sub>8</sub> C <sub>8</sub> C <sub>8</sub> C <sub>8</sub> <u>TTT TTT</u> CCC CCC CTT TTC TTT T 3' (SEQ ID NO:17)
10	TFO F <sub>1</sub> (pseudo- circle)	5' GCA TGC CCT TTT CTT TTC CCC CCT <u>TTT TTC</u> CCC CCT TTT CTT TTC C GCA TGC 3' (SEQ ID NO:38)

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- 32 -

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

(i) APPLICANT: Moelling, Karin

(ii) TITLE OF INVENTION: Inhibition Of Viruses By Antisense Oligomers Capable Of Binding To Polypurine-Rich Tract Of Single-Stranded RNA Or RNA-DNA Hybrids

(iii) NUMBER OF SEQUENCES: 42

## (iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: Woodcock Washburn Kurtz  
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(B) STREET: One Liberty Place - 46th Floor

(C) CITY: Philadelphia

(D) STATE: PA

(E) COUNTRY: USA

(F) ZIP: 19103

## (v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: PC-DOS

(D) SOFTWARE: WORDPERFECT 5.1

## (vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: n/a

(B) FILING DATE: Herewith

(C) CLASSIFICATION:

## (vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/954,184

(B) FILING DATE: 29-SEP-1992

## (viii) ATTORNEY/AGENT INFORMATION:

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## (ix) TELECOMMUNICATION INFORMATION:

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## (2) INFORMATION FOR SEQ ID NO: 1:

## (i) SEQUENCE CHARACTERISTICS:

- 33 -

- (A) LENGTH: 16
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear
- (iv) ANTI-SENSE: No
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:  
AAAAGAAAAG GGGGGA 16
- (2) INFORMATION FOR SEQ ID NO: 2:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 26
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Linear
  - (iv) ANTI-SENSE: No
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:  
AAAAGAAAAG GGGGGACTGG AAGGCG 26
- (2) INFORMATION FOR SEQ ID NO: 3:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 25
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Linear
  - (iv) ANTI-SENSE: Yes
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:  
TTTTCTTTTC CCCCTGACC TTCCC 25
- (2) INFORMATION FOR SEQ ID NO: 4:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 25
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Linear
  - (iv) ANTI-SENSE: Yes
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:  
TTTTGTTTTG GGGGTGTGG TTGGG 25
- (2) INFORMATION FOR SEQ ID NO: 5:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 16

- 34 -

- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TTTTCTTTTC CCCCCT 16

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TTTTGTTTTG GGGGGT 16

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

AAAAGAAAAG GGGGGACTGG AAGGG 25

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CCCTTCCAGT CCCCCCTTTT CTTTT 25

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40
- (B) TYPE: Nucleic Acid

- 35 -

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TGAATTAGCC CTTCCAGTCC CCCCTTTTCT TTTAAAAAGT 40

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

AGTCCCCCT TTTCTTTTAA 20

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCGCCCAGGC CACGCCTCCC 20

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

TTTTCTTTTG GGGGGTTTGG TTGGGTTTTC CCTTCCAGTC CCCCCTTTTC 50  
TTTTT 54

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54

(B) TYPE: Nucleic Acid

- 36 -

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

TGTGTGTGTG TGTGTGTGTG TGTGTTTTTC CCTTCCAGTC CCCCCTTTTC 50  
TTTT 54

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

TTTTATTTTA GGGGATTGG TTGGGTTTTTC CCTTCCAGTC CCCCCTTTTC 50  
TTTT 54

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

TTTTCTTTTG GGGGGTCTGG TTGGGTTTTTC CCTTCCAGTC CCCCCTTTTC 50  
TTTT 54

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

TTTTGTTTTG GGGGGTTTTT TCCCCCTTT TCTTTT 36

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- 37 -

- (A) LENGTH: 36
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear
- (iv) ANTI-SENSE: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:  
TTTTCTTTTC CCCCTTTTTT TCCCCCTTT TCTTTT 36
- (2) INFORMATION FOR SEQ ID NO: 18:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 36
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Linear
  - (iv) ANTI-SENSE: Yes
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:  
GGGGGGTTTG GTTGGGTTTT CCAACAAA CCCCC 36
- (2) INFORMATION FOR SEQ ID NO: 19:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 35
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Linear
  - (iv) ANTI-SENSE: Yes
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:  
GGGGGGTTTG GTTGGGTTTC CCTTCCAGTC CCCCC 35
- (2) INFORMATION FOR SEQ ID NO: 20:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 36
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Linear
  - (iv) ANTI-SENSE: Yes
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:  
GGGGGGTTTG GTTGGGTTTT TTCCCTTCCA GTCCCC 36
- (2) INFORMATION FOR SEQ ID NO: 21:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 35

- 38 -

- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear
- (iv) ANTI-SENSE: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:  
AGGGGATTTG GTTGGGTTTT CCCTTCCAGT CCCCC 35
- (2) INFORMATION FOR SEQ ID NO: 22:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 35
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Linear
  - (iv) ANTI-SENSE: Yes
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:  
GGGGGGTTTG GTTGGGTTTT CCCTTCCAGT CCCCC 35
- (2) INFORMATION FOR SEQ ID NO: 23:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 27
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Linear
  - (iv) ANTI-SENSE: Yes
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:  
CCCCCTCTCT TCAAAGAGA AGGGGGG 27
- (2) INFORMATION FOR SEQ ID NO: 24:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 28
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Linear
  - (iv) ANTI-SENSE: Yes
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:  
CCCCCTTCTT CAAAAGAGAG GTTGGGGG 28
- (2) INFORMATION FOR SEQ ID NO: 25:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 33
    - (B) TYPE: Nucleic Acid



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- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear
- (iv) ANTI-SENSE: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:  
GGGGACTGGG AGGGTTTTCC CTTCCAGTCC CCC 33
- (2) INFORMATION FOR SEQ ID NO: 26:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 54
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Linear
  - (iv) ANTI-SENSE: Yes
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:  
TTTTCTTTTG GGGGGTTTGG TTGGGTTTTC CCTTCCAGTC CCCCCTTTTC 50  
TTTT 54
- (2) INFORMATION FOR SEQ ID NO: 27:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 36
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Linear
  - (iv) ANTI-SENSE: Yes
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:  
GGGGGGTTTG GTTGGGTTTT CCCTTCCAGT CCCCCC 36
- (2) INFORMATION FOR SEQ ID NO: 28:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 36
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Linear
  - (iv) ANTI-SENSE: Yes
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:  
GGGGGGACTG GTTGGGTTTT CCCTTCCAGT CCCCCC 36
- (2) INFORMATION FOR SEQ ID NO: 29:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 16
    - (B) TYPE: Nucleic Acid

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- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear
- (iv) ANTI-SENSE: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:  
GGGGGGTTGT CCCCCC 16
- (2) INFORMATION FOR SEQ ID NO: 30:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 36
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Linear
  - (iv) ANTI-SENSE: Yes
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:  
GGGGGGTTTG GAAGGGTTTT CCCTTCCAGT CCCCCC 36
- (2) INFORMATION FOR SEQ ID NO: 31:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 36
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Linear
  - (iv) ANTI-SENSE: Yes
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:  
GGGGGGACTG GAAGGGTTTT CCCTTCCAGT CCCCCC 36
- (2) INFORMATION FOR SEQ ID NO: 32:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 24
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single
    - (D) TOPOLOGY: Linear
  - (iv) ANTI-SENSE: Yes
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:  
TTTGTTGGG TTTCCCTTC CAGT 24
- (2) INFORMATION FOR SEQ ID NO: 33:
  - (i) SEQUENCE CHARACTERISTICS:
    - (A) LENGTH: 36
    - (B) TYPE: Nucleic Acid
    - (C) STRANDEDNESS: Single

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- (D) TOPOLOGY: Linear
- (iv) ANTI-SENSE: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:  
GGGGGGNNNG GTTGGGTTTT CCCTTCCAGT CCCCCC 36
- (2) INFORMATION FOR SEQ ID NO: 34:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 36
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear
- (iv) ANTI-SENSE: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:  
GGGGGGTTTG GNNGGGTTTT CCCTTCCAGT CCCCCC 36
- (2) INFORMATION FOR SEQ ID NO: 35:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 35
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear
- (iv) ANTI-SENSE: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:  
GGGGGGTTTG GTTGNNTTTT CCCTTCCAGC CCCCC 35
- (2) INFORMATION FOR SEQ ID NO: 36:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 45
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear
- (iv) ANTI-SENSE: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:  
GGGGGGTTTG GTTGGGTTTT CCCTTCCAGT CCCCCCTTTT CTTTT 45
- (2) INFORMATION FOR SEQ ID NO: 37:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 36
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Unknown

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(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

UUUUCUUUUC CCCCCTTTT TCCCCCTTT TCTTTT

36

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 52

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Unknown

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

GCATGCCCTT TTCTTTTCCC CCCTTTTTTC CCCCCTTTTC TTTTCCGCAT  
GC 52

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

GGGTTGGTGT GGGGGGTTTT GTTTT

25

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

TGGGGGGTTT TGTTTT

16

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

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- (D) TOPOLOGY: Linear
- (iv) ANTI-SENSE: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:  
UUUUGUUUUG GGGGGUGUGG UUGGG 25
- (2) INFORMATION FOR SEQ ID NO: 42:
- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 25
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear
- (iv) ANTI-SENSE: Yes
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:  
CCCTTCCAGT CCCCCCTTTT CTTTT 25

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**What is claimed is:**

1. A method of inhibiting a virus with which a vertebrate is infected which replicates via an RNA template having a polypurine-rich tract (PPT) and which forms an RNA-DNA  
5 hybrid during replication, comprising administering to said vertebrate an oligonucleotide, or derivatives for increasing bioavailability thereof, capable of binding to at least a portion of said PPT of said RNA-DNA hybrid and inhibiting replication of said virus, in an amount effective for inhibit-  
10 ing said replication.
2. A method of inhibiting a virus with which a vertebrate is infected which replicates via an RNA template and which forms a single-stranded RNA having a polypurine tract (PPT), comprising administering to said vertebrate an oligomer,  
15 or derivatives for increasing bioavailability thereof, capable of binding to at least a portion of said PPT in said single-stranded RNA and inhibiting replication of said virus, in an amount effective for inhibiting said replication.
3. The method of claim 1 wherein said virus is a  
20 retrovirus or a hepadnavirus.
4. The method of claim 3 wherein said retrovirus is HIV-1.
5. The method of claim 2 wherein said virus is a retrovirus or a hepadnavirus.
- 25 6. The method of claim 5 wherein said retrovirus is HIV-1.

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7. The method of claim 1 wherein said oligonucleotide comprises a sequence selected from the group consisting of TTTTCTTTTCCCCCTGACCTTCCC (SEQ ID NO: 3),  
AAAAGAAAAGGGGGGACTGGAAGGG (SEQ ID NO: 7),  
5 CCCTTCCAGTCCCCCCTTTTCTTTT (SEQ ID NO: 8),  
TTTTGTTTTGGGGGGTGTGGTTGGG (SEQ ID NO: 4),  
TTTTGTTTTGGGGGGT (SEQ ID NO: 6), and  
UUUUGUUUUGGGGGGUGUGGUUGGG (SEQ ID NO: 41).
8. The method of claim 2 wherein said oligonucleotide is selected from the group consisting of  
10 AGTCCCCCCTTTTCTTTTAA (SEQ ID NO: 10) and  
TGAATTAGCCCTTCCAGTCCCCCCTTTTCTTTTAAAAAGT (SEQ ID NO: 9).
9. The method of claim 1 wherein said oligonucleotide comprises a deoxyribonucleotide, or derivative for  
15 increasing bioavailability thereof.
10. The method of claim 2 wherein said oligonucleotide comprises a deoxyribonucleotide or a derivative for increasing bioavailability thereof.
11. The method of claim 1 wherein said oligonucleotide comprises from about 8 to about 40 nucleotides.  
20
12. The method of claim 2 wherein said oligonucleotide comprises from about 8 to about 40 nucleotides.
13. The method of claim 1 wherein said oligonucleotide comprises from about 16 to about 26 nucleotides.
- 25 14. The method of claim 2 wherein said oligonucleotides comprises from about 16 to about 26 nucleotides.
15. The method of Claim 1 or 2 wherein said derivatives comprise oligonucleotides having one or more of the terminal phosphodiester bonds modified phosphorothioates.

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16. The method of Claim 1 or 2 wherein said derivatives comprise oligonucleotides having an amino group at at least one terminal end.

17. An oligonucleotide comprising a sequence  
5 selected from the group consisting of  
TTTTCTTTTCCCCCTGACCTTCCC (SEQ ID NO: 3),  
AAAAGAAAAGGGGGGACTGGAAGGG (SEQ ID NO: 7),  
CCCTTCCAGTCCCCCTTTTCTTTT (SEQ ID NO: 8),  
TTTTGTTTTGGGGGGTGTGGTTGGG (SEQ ID NO: 4),  
10 TTTTGTTTTGGGGGGT (SEQ ID NO: 6), and  
UUUUGUUUUGGGGGGUGUGGUUGGG (SEQ ID NO:41)  
or derivatives for increasing bioavailability thereof.

18. An oligonucleotide comprising  
TTTTGTTTTGGGGGGTGTGGTTGGG (SEQ ID NO: 4) or derivatives for  
15 increasing bioavailability thereof.

19. An oligonucleotide comprising  
UUUUGUUUUGGGGGGUGUGGUUGGG (SEQ ID NO:41) or derivatives for  
increasing bioavailability thereof.

20. An oligonucleotide capable of forming a triplex  
20 with a single-stranded nucleic acid.

21. The oligonucleotide of claim 20 wherein said  
single-stranded nucleic acid is viral RNA.

22. The oligonucleotide of claim 21, wherein said  
RNA has a polypurine-rich tract is involved in replication.

23. An oligonucleotide having the formula 5'-A-B-C-  
25 3'

wherein A comprises a segment of nucleotides  
complementary in parallel orientation to a polypurine-rich  
tract of a viral nucleic acid having said tract;



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wherein B comprises a linker of at least two nucleotides; and

wherein C comprises a segment of nucleotides complementary to said tract in anti-parallel orientation.

5           24. The oligonucleotide of claim 23 wherein A comprises ribonucleotides.

25. The oligonucleotide of claim 23 wherein A comprises deoxyribonucleotides.

26. The oligonucleotide of claim 23 wherein said  
10 oligonucleotide comprises a derivative form for increasing the bioavailability thereof.

27. The derivative form of claim 26 wherein said oligonucleotide possesses at least two phosphorothioate bonds.

28. The derivative form of claim 27 wherein said  
15 phosphorothioate bonds are at terminal ends of said oligonucleotide.

29. A composition of matter comprising  
an oligonucleotide, or derivative for increasing  
bioavailability thereof, having the formula 5'-A-B-C-3'

20           wherein A comprises a segment of nucleotides complementary in parallel orientation to a polypurine-rich tract of a viral nucleic acid having said tract;

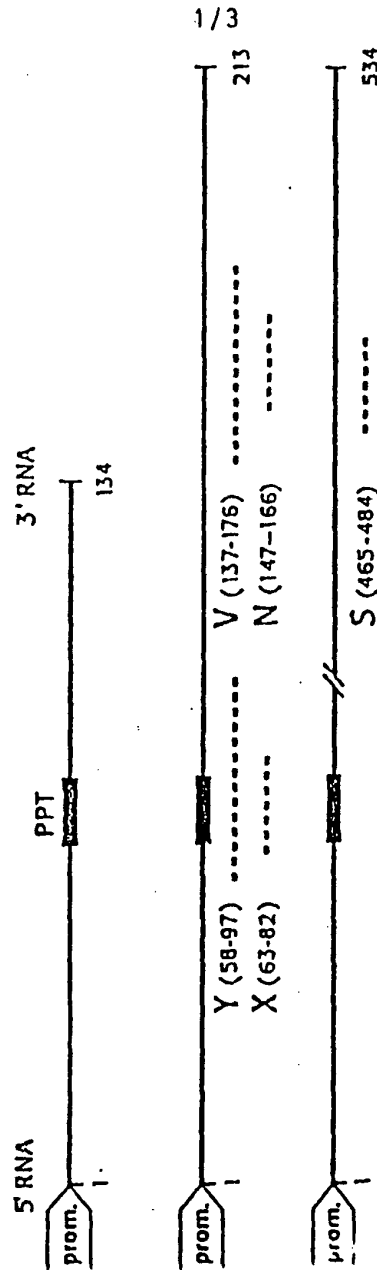
wherein B comprises a linker of at least two nucleotides; and

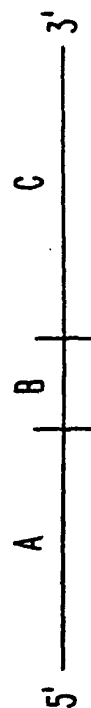
25           wherein C comprises nucleotides complementary to said tract in anti-parallel orientation; and  
a vehicle for enhancing the bioavailability of said composition in vivo.

30. The composition of matter of Claim 29 wherein  
30 said vehicle comprises a liposome.

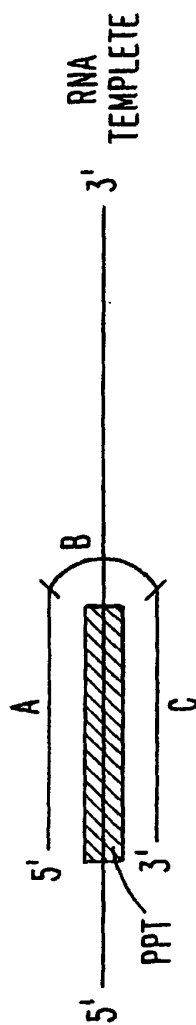
Page missing at the time of publication

FIGURE 1

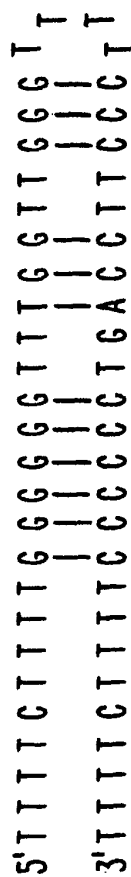




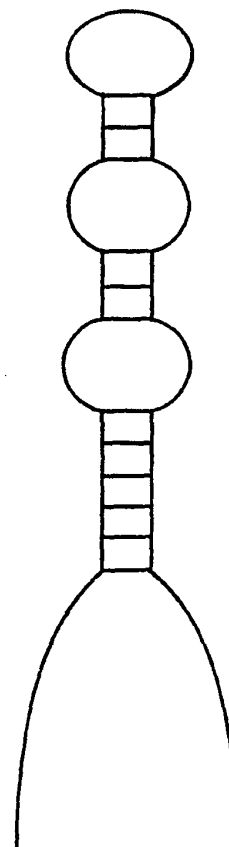
***Fig. 2a***



***Fig. 2b***

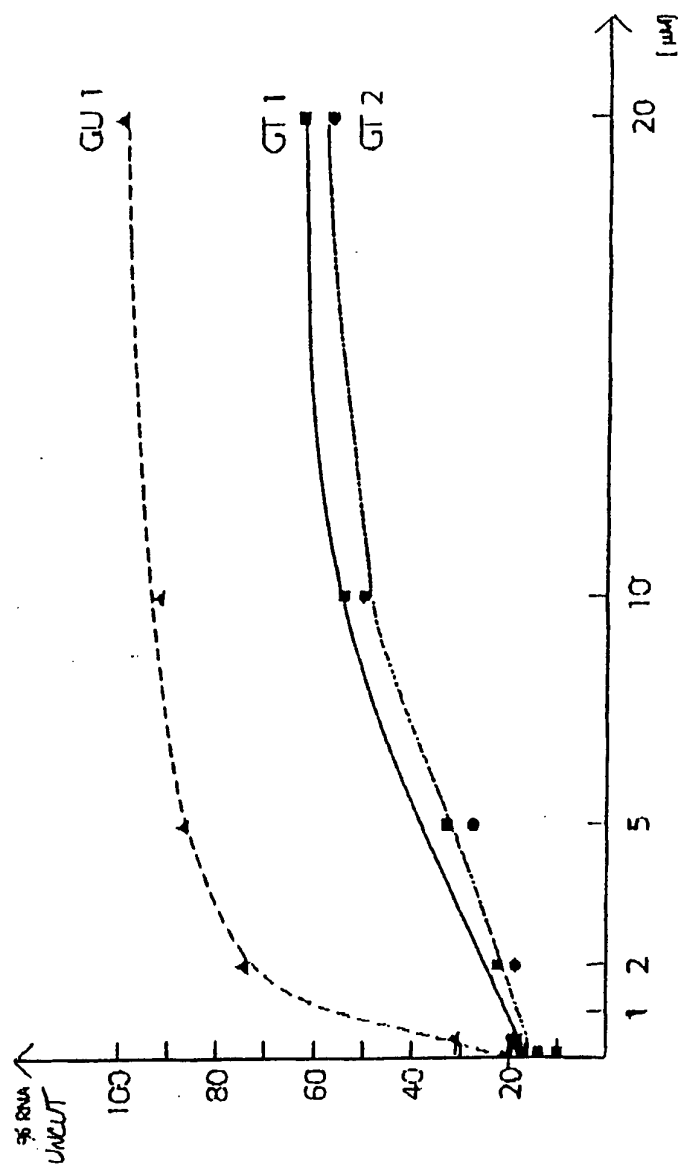


***Fig. 3a***



***Fig. 3b***

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4  
FIGURE

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US93/09300

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A01N 43/04; A61K 31/70, 31/795; C07H 21/00, 21/02, 21/04  
US CL : 514/44; 536/23.1, 24.1, 24.5

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/44; 536/23.1, 24.1, 24.5; 935/2, 6, 34

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS,  
search terms: polypurine, triplex, linker

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Chemical Reviews, Volume 90, No. 4, issued June 1990, E. Uhlmann et al., "Antisense Oligonucleotides: A New Therapeutic Principle", pages 543-584, especially pages 569-572, 757, and 577-578.	1-30
Y	Journal of Biological Chemistry, Volume 265, No. 18, issued 25 June 1990, H. E. Huber et al., "Processing of the Primer for Plus Strand DNA Synthesis by Human Immunodeficiency Virus 1 Reverse Transcriptase", pages 10565-10573, especially pages 10568, 10572.	1-30

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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*O document referring to an oral disclosure, use, exhibition or other means		
*P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

12 November 1993

Date of mailing of the international search report

02 DEC 1993

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## INTERNATIONAL SEARCH REPORT

 International application No.  
 PCT/US93/09300

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, Volume 83, issued June 1986, P. C. Zamecnik et al., "Inhibition of Replication and Expression of Human T-cell Lymphotropic Virus Type III in Cultured Cells by Exogenous Synthetic Oligonucleotides Complementary to Viral RNA", pages 4143-4146, especially page 4145.	1-30
Y	Proceedings of the National Academy of Sciences, Volume 88, issued September 1991, E. H. Postel et al., "Evidence that a Triplex-Forming Oligodeoxyribonucleotide Binds to the <u>c-myc</u> Promoter in HeLa Cells, Thereby Reducing <u>c-myc</u> mRNA Levels", pages 8227-8231, especially page 8230.	1, 3, 4, 7, 9, 11, 13, 17-30
Y	Science, Volume 245, issued 18 August 1989, L. J. Maher III et al., "Inhibition of DNA Binding Proteins by Oligonucleotide-Directed Triple Helix Formation", pages 725-730, especially pages 728, 730.	1-30
Y	US, A, 5,138,045 (Cook et al.) 11 August 1992, col. 3, lines 34-59.	16
Y	Biochemistry, Volume 30, No. 14, issued 1991, A. Ono et al., "DNA Triplex Formation of Oligonucleotide Analogues Consisting of Linker Groups and Octamer Segments That Have Opposite Sugar-Phosphate Backbone Polarities", pages 9914-9921, especially page 9915.	23-30
Y	Current Topics in Microbiology and Immunology, Volume 141, issued 1988, S. L. Loke et al., "Delivery of <u>c-myc</u> Antisense Phosphorothioate Ologodeoxynucleotides to Hematopoietic Cells in Culture By Liposome Fusion: Specific Reduction in <u>c-myc</u> Protein Expression Correlates With Inhibition of Cell Growth and DNA Synthesis", pages 282-289, especially page 288.	30